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Biotechnological production of recombinant tissue plasminogen activator protein (reteplase) from transplastomic tobacco cell cultures.

Diego Hidalgo[a†], *Maryam Abdoli Nasab*[b], *Mokhtar Jalali-Javaran*[c], *Roque Bru-Martínez*[d], *Rosa M. Cusidó*[a], *Purificación Corchete*[e], *Javier Palazon*[a]*

[a]*Laboratori de Fisiologia Vegetal, Facultat de Farmacia, Universitat de Barcelona, Av. Joan XXIII sn, 08028 Barcelona, Spain*

[b]*Department of Biotechnology, Institute of Science, High Technology and Environmental Science, Graduate University of Advanced Tecnology, P.O. Box 76315-117, Kerman, Iran*

[c]*Department of Plant Breeding, Faculty of Agriculture, Tarbiat Modares University, P.O. Box 14115-336, Tehran, Iran*

[d]*Plant Proteomics and Functional Genomics Group, Department of Agrochemistry and Biochemistry, Faculty of Science, University of Alicante, Alicante, Spain*

[e]*Department of Botany and Plant Physiology, Campus Miguel de Unamuno, University of Salamanca, E-37007 Salamanca, Spain*

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ABSTRACT

Transplastomic plants are a system of choice for the mass production of biopharmaceuticals due to the polyploidy of the plastid genome and the low risk of pollen-mediated outcrossing because of maternal inheritance. However, as field-grown plants, they can suffer contamination by agrochemicals and fertilizers, as well as fluctuations in yield due to climatic changes and infections. Tissue-type plasminogen activator (tPA), a protein used to treat heart attacks, converts plasminogen into plasmine, which digests fibrin and induces the dissolution of fibrin clots. Recently, we obtained transplastomic tobacco plants carrying the K2S gene encoding truncated human tPA (reteplase) with improved biological activity, and confirmed the presence of the target protein in the transgenic plant leaves. Considering the advantages of plant cell cultures for biopharmaceutical production, we established a cell line derived from the K2S tobacco plants. The active form of reteplase was quantified in cultures grown in light or darkness, with production 3-fold higher in light.

1. Introduction

Biopharmaceuticals based on proteins, antibodies or nucleic acids are increasingly being used for disease treatment. Although only about 60 peptides have been approved by the US FDA to date, more than 140 are under clinical study and by 2020 the global sales of biopharmaceuticals are expected to be worth over $US 278.2 billion (Santos et al., 2016).

Tissue type plasminogen activator (tPA), which induces the dissolution of fibrin clots by converting the zymogen plasminogen into the serine protease plasmin, is a clinically useful thrombolytic agent (Clark, 2001) and a target for biotechnological production. tPA has five domains, N terminal finger, epidermal growth factor, serine protease, Kringle 1 and Kringle 2 (Youchung et al., 2003). The active part of tPA, the thrombolytic Kringle 2 domain, serine protease domain, two functional regions of proteasa (176-527 amino acid residues), plus the 1 to 3 amino acids of the N-terminal is known as the truncated human tissue plasminogen activator (K2S, reteplase), which has a longer plasma half-life and higher fibrinolytic activity than tPA (Nordt and Bode, 2003).

The main biotechnological systems for the production of recombinant biopharmaceuticals are based on microorganism cultures such as *Escherichia coli* and yeast at bioreactor level, while large proteins are generally produced by mammalian cell platforms (Demain and Vaishnav, 2009). Molecular farming also has been utilized for biopharmaceutical production, because transgenic plants only need water, minerals and sunlight for growth, but a drawback of the system, according to the US Agricultural Department, is a lack of guaranteed transgene contention and the risk of contamination of the human food chain if edible plant species are used as the host (Wilson and Roberts, 2012). Moreover, transgenic crops used for the production of heterologous

proteins are exposed to agrochemicals and fertilizers in the field, while variable culture conditions and the impact of bacterial and fungal infections can lead to fluctuations in yield (Hellwig et al., 2004).

As an alternative production system, plant cell cultures share the capacity of transgenic crops for proper protein folding and can assemble complex recombinant proteins. They also have similar advantages to bioreactor systems based on microorganisms and mammalian cells, as they avoid transgene dissemination and provide controlled and sterile growth conditions, chemically defined culture media, and compliance with pharmaceutical good manufacturing practices, ensuring the biosafety and productivity of the system (Demain and Vaishnav, 2009; Santos et al., 2016). Furthermore, they allow proteins to be manufactured in days or weeks, rather than the months or years required when depending on the growth cycle of a whole plant (Doran, 2000).

Several factors affecting transgenic crops, such as climate, soil quality, season, day length and weather, are not issues for biotechnological platforms based on plant cell cultures. Additionally, the secretion of heterologous proteins into the culture medium simplifies downstream processing and protein purification (Pham et al., 2012). The first human recombinant protein approved in the US and other countries was Taliglucerase alfa, a modified glucocerebrosidase enzyme used to treat Gaucher's disease, produced by Protalix Biotherapeutics in the ProCellEx® platform based on carrot cell cultures (Tekoah et al., 2015).

Transplastomic plants have been targeted for the production of biopharmaceuticals due to the high number (approx. 100) of chloroplasts per plant cell, the high copy number (approx. 10000) of the plastid genome, as well as the maternal mode inheritance, though low-level leakages of transgenes in pollen may occur (Bock, 2014). Transplastomic technology has enhanced field-grown plant resistance to herbicides and plagues and has

been used for the production of recombinant proteins. However, the derived cell cultures have been scarcely applied for the biotechnological production of heterologous proteins (Block, 2007). Examples of biopharmaceuticals produced in transplastomic tobacco plant cell cultures include the phage-derived endolysins, used as an antibiotic against pneumonia (Oey et al., 2009), camelid antibodies (Lentz et al., 2012), the transforming growth factor (TGFβ3), a cytokine-type protein (Gisby et al., 2011), and fragment C of tetanus toxin (TetC). The latter was accumulated up to 7 mg/L, but when the transplastomic cell suspension was cultured in a temporary immersion bioreactor (TIB), regenerated shoots achieved a TetC production of 95 mg/L (Michoux et al., 2011).

Recently, our group obtained tobacco transplastomic plants harboring the K2S gene driven by the promotor Prrn for the production of the truncated human tissue plasminogen activator (K2S, reteplase), which is one of the most important pharmaceutical recombinant proteins, widely used to break down blood clots through the conversion of plasminogen to plasmin (Abdoli-Nasab et al., 2013). The purification system of reteplase has been recently optimized, reaching a production of up to 30.6 µg/100 mg fresh weight of leaf tissue (Abdoli-Nasab et al., 2016). Taking into account the potential advantages of plant cell cultures for the production of biopharmaceuticals, in this work we demonstrate for the first time the capacity of cell suspension cultures derived from K2S transplastomic tobacco plants to produce the bioactive target peptide.

2. Material and Methods

2. 1. Plant material

In this work, we utilized seeds from homoplastic plants carrying the K2S gene driven by the promotor Prrn obtained as described by Abdoli-Nasab et al. (2013). Sterile seeds were germinated on solidified MS (Murashige and Skoog, 1962) medium supplemented with 500 mg/L spectinomycin, in Magenta vessels (SIGMA). The *in vitro* plantlets were cultivated in a climate chamber at 25ºC under a 16 h photoperiod and an approximate light intensity of 60 μmol m^{-2} s^{-1}.

2.2. Initiation and maintenance of the transgenic cell suspension

Leaf discs of young K2S plants were cultivated in darkness or under light conditions for callus induction in solid MS medium (Murashige and Skoog, 1962) supplemented with 2.14 mg/L of naphthalene acetic acid in combination with 0.215 mg/L of kinetin (Piñol et al., 1985) and 500 mg/L of spectinomycin (Fig. 1). After several subcultures, 30 g of friable calli were placed in 300 mL of liquid MS medium with the same hormones and antibiotic to obtain a fine cell suspension, which was subcultured every 12 days, shaken at 115 rpm and maintained at 25 °C in darkness or light conditions.

2.3. qPCR analysis

Expression of the *K2S* gene in the cell suspension was verified by qPCR. The elongation factor 1 α (EF-1 α) as the nuclear-encoded reference (Schmidt et al., 2010) and the accD-like plastid-encoded (Lee et al., 2004) were used for gene normalization. The stability of the housekeeping gene expression was analyzed by calculating the coefficient of variation (CV), as previously described (Exposito-Rodriguez et al., 2008). Total RNA from the plant material was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA). For the qRT-PCR, cDNA was prepared from RNA treated with DNase I (Invitrogen, Carlsbad, CA) and synthesized with SuperScript IV reverse transcriptase

(Invitrogen, Carlsbad, CA). qRT-PCR was performed using the iTAqTM universal SYBR Green Supermix (BioRad, Hercules, CA, EEUU) in a 384-well platform system (LightCycler_480 Instrument; Roche), and each sample was run in triplicate, under the following conditions: 95 °C for 2 min, 40 cycles (95 °C, 10 s; 60 °C, 20 s; 72 °C, 20 s) followed by a melting curve. Gene-specific primers were designed with Primer-BLAST (Table 1).

2.4. Protein extraction

Total soluble protein (TSP) was extracted as described by Wang et al. (2005) with slight modifications. Briefly, 4 g of the harvested cell suspension was ground in liquid nitrogen and placed in a 15 mL tube, which was filled with 10% trichloroacetic acid / acetone, and centrifuged at 10000 g for 5 min (4°C). The supernatant was removed, washed with 0.1 M ammonium acetate / 80% methanol and centrifuged. The pellet was washed with 80% acetone, and air-dried at room temperature for 10 min. The tube with the pellet was filled with phenol/SDS buffer (Wang et al., 2005), mixed thoroughly and incubated for 5 min on ice. After centrifugation, the upper phase was recovered in a new tube, which was filled with 0.1 M ammonium acetate / 80% methanol, incubated over night at -20°C and then centrifuged at 10000 g for 10 min (4°C). Finally, the pellet was washed once with 100% methanol and once with 80% acetone and air-dried. The protein was resuspended in 6 M urea and quantified using the RC DC Protein Assay Kit II (Bio-Rad, CA, USA).

Native proteins were extracted using finely ground powder under liquid nitrogen. Per 5 g of cells, 10 mL of cold buffer solution was used (buffer: 250 mM sucrose, 50 mM HEPES, 5% glycerol, 10 mM $Na_2O_5S_2$, 1% PVP, 10 mM ascorbic acid and 100 mM PMSF, pH=7.5). The mixture was thoroughly homogenized and centrifuged at 12000 g for 15 min (4°C). Finally, the supernatant was purified for further analysis.

2.5. Protein purification

For purification of the recombinant protein, a His Gravitrap™ TALON® (GE HealthcareBio-Sciences AB, Uppsala, Sweden) was used following the manufacturer's instruction. The eluted protein was concentrated by Amicon® Ultra-2 (30K) Centrifugal Filter Devices (Millipore Corp., Bedford, MA), and the buffer exchange was carried out with buffer phosphate (50 mM sodium phosphate, 300mM NaCl, pH=7.4). Finally, the quantification was done by the RC DC Protein Assay Kit II (Bio-Rad, CA, USA).

2.6. SDS-PAGE and sample preparation for mass spectrometry analysis

The TSP extracted from the cell suspension was placed (100 µg) in 12% acrylamide gel followed by staining with Coomassie brilliant blue (Laemmli, 1970). The gel bands located at 48 kDa were processed as described by Gundry et al. (2010). For digestion, sequencing grade modified trypsin (V5111, Promega, Madison, WI) was used and samples desalted with PepClean™ C-18 Spin Columns (Thermo Fisher Scientific, Rockford, IL) according to the manufacturer's recommendations.

2.7. Liquid chromatography-mass spectrometry

For protein identification, MS and MS/MS data were acquired in an Agilent XCT plus ion trap mass spectrometer with a ChipCube interface fed by an Agilent 1100 series nanopump HPLC system (Martínez-Esteso et al., 2011). The four most intense precursor ions in the MS scans were selected for MS/MS and then passed to an active exclusion list released after 1min. Raw data were converted into a peak list with the extraction tool of SpectrumMill Proteomics Workbench (SMPW) (Agilent). The reduced data set was searched against the Swissprot forward and reversed protein database without taxonomical restrictions in the identity mode with the MS/MS search tool of SpectrumMill Proteomics Workbench, using the following parameters: trypsin,

up to 1 missed cleavages, fixed modification carbamidomethylation of cystein, the variable modification oxidation of methionine, and a mass tolerance of 2.5 Da for the precursor and 0.7 Da for product ions. Peptide hits were validated first in the peptide mode and then in the protein mode according to the score settings recommended by the manufacturer.

The MRM selector tool of SMPW was used to generate transition lists from validated peptide identifications by selecting the five most intense precursor/product ion pairs for each target peptide above the precursor m/z, and calculating the collision energy for each precursor ion.

The MRM experiments were performed on an Agilent's standard flow LC-MRM-MS platform (Agilent Technologies, Palo Alto, CA) consisting of a 1290 Infinity UHPLC interfaced to a 6490 triple quadrupole mass spectrometer via a JetStream ESI source. Protein sample digests were separated using an Advance Bio Peptide Map 2.1*150mm 2.7 µm column thermostated at 50ºC at 0.4 mL/min flow rate over a 6-min run along a linear gradient from 0 to 70 % of solvent B consisting of 0.1% FA in 90% ACN. Source parameters were: 3000-3500 V capillary voltage, 15 L/min N_2 gas flow, 150ºC gas temperature. Each transition was acquired at unit resolution in standard mode for 10ms dwell time at 3 duty cycles per second.

A project was created in the open source application Skyline (MacLean et al., 2010) using the sequence of identified peptides and selecting manually the previously generated transitions, in order to import, visualize, refine the acquisition method and compare the LC-MRM runs.

2.8. Activity assay

The protein assay was performed with a tissue-type plasminogen activator (tPA) Human Chromogenic Activity Assay Kit (ab108905, abcam, Cambridge, UK). This assay measures the ability of tPA to activate the plasminogen to plasmin. The amount of plasmin produced is quantitated using a specific substrate releasing a yellow *p*-nitroaniline chromophore. Briefly, human plasminogen, plasmin substrate and tPA standard were prepared following the manufacturer's instructions. Then, a standard curve was prepared in triplicate by serial dilution, considering 7 points (40, 10, 2.5, 0.625, 0.156, 0.039 and 0 IU mL^{-1}). Next, a mixture of specific diluent, plasminogen and plasmid substrate (60, 10 and 10 µL respectively) was distributed in a 96-well microplate. Finally, 20 µL of standard points or samples were added, incubated at 37 °C and the absorbance was periodically read at 405 nm for up to 9 h, according to the manufacturer's recommendation.

3. Results and Discussion

3.1 Establishment of transgenic cell suspension cultures and K2S gene expression.

After 2 months of the initial callus induction, sufficient material was obtained to establish the cell suspension. A characteristic green color was observed in the callus and cell suspension grown in light (Fig. 1 A, B). After 12 days of culture, samples from the cell suspension growing in light or darkness were taken for the planned analyses. Their growth capacity was measured as a growth index (harvested fresh weight / inoculum fresh weight, GI) and biomass productivity (r_x). Light was found not to affect the biomass production of the system, and in both conditions, the cell cultures reached a GI > 3, which represents a r_x > 18.5 g $L^{-1}d^{-1}$ (Fig. 2), a growth capacity very similar to the

untransformed tobacco cell lines (data not shown). These results show the high capacity of the system based on tobacco suspension cultures to produce biomass, which was not affected by the K2S gene expression. Similar results for growth capacity were reported previously for tobacco cell cultures genetically designed for the heterologous production of the *t*-resveratrol derivative, *t*-piceatannol (Hidalgo et al., 2017), and for the production of scopolamine in transgenic tobacco cell lines carrying the *Hn*H6H gene (Moyano et al., 2007).

In order to normalize the gene expression data more precisely in the qPCR analyses, cp values of housekeeping genes (EF1 and accD) were used, in combination or separately, to calculate and compare the CV between them, as described in Material and Methods. The results, CV_{EF1}= 0.0247 < $CV_{EF1\&accD}$= 0.0307 < CV_{accD} = 0.0513, demonstrated that the accD gene expression was affected by light and was therefore not useful for this experiment, while EF1 proved more stable under both conditions, giving the smallest CV value, which indicates less variability than shown by the combination of EF1 and accD or accD (Table 2 and Figure 1 C).

In contrast with the growth capacity, the analysis of the K2S gene expression showed significant differences according to the conditions, with 4.6-fold higher gene expression when the cell line was cultured under light. These results could be related to the green color of this cell suspension, which is probably caused by a higher degree of chloroplast organization and/or their number per cell. Recently, Barreto et al. (2017) developed transgenic calli for the heterologous production of the fragment C of tetanus toxin (TetC), and reported a positive correlation between green shoot development and TetC yield when the callus was cultivated in a temporary immersion culture.

3.2 Characterization of rtPA *heterologous protein by Mass Spectrometry*

The acrylamide gel bands corresponding to the predicted molecular weight of the K2S protein were successfully submitted to qualitative analysis. A method was previously established to detect 7 different reteplase peptides, 3 of which were proteotypic (Fig. 3), i.e. they were exclusive to reteplase and with no homology with related species (Fig.4). Each peptide was detected with at least 3 transitions, which gives specificity to the analysis. In all the samples from transgenic material overexpressing the K2S gene, peptides associated with reteplase were found and as expected, the signals were not detected in the wild type cell suspension. In all samples, the most intensive signals were detected for the peptides GGLFADIASHPWQAAIFAK and VYTAQNPSAQALGLGK (Fig. 5 A), at a retention time of 3.4 min and 2.0 min respectively (Fig. 5 B). The transitions of the peptide GGLFADIASHPWQAAIFAK were detected both in samples kept in the dark and in light without significant quantitative differences probably because the transitions for this peptide give rise to many unspecific signals in the tobacco proteome background. Transitions of the peptide VYTAQNPSAQALGLGK were well above noise in light treated samples while only noise could be detected in darkness treated samples. The later is consistent with the higher plastid abundance and the K2S expression levels in light conditions, thus validating the suitability monitoring VYTAQNPSAQALGLGK by MRM as a surrogate peptide of K2S. The results confirmed the capacity of the K2S transgenic cell line to biosynthesize the target biopharmaceutical.

3.3 Determination of the protein content and activity assay.

As the reteplase was polyhistidine-tagged (Abdoli et al. 2013), the recombinant protein expressed in chloroplasts was estimated by measuring the TSP before purification and after concentration of the eluted protein from the column, as described in Material and

Methods. The rtPA content reached in the tobacco cell suspension when cultivated in darkness was 0.083 % of the TSP, rising to 0.277% in light conditions, which represents an increase of more than 3-fold. These results are consistent with the MRM signal intensities for the peptide VYTAQNPSAQALGLGK and the production values were lower than those obtained in the transplantomic mother plant (approx. 1% of TSP) (Abdoli Nasab et al., 2016), but in that case the protein was harvested after 8 weeks rather than the 2 weeks of the cell suspension culture period. In accordance with previous reports (Michoux et al., 2011; Hong et al., 2002; Hellwig et al., 2004; Magnuson et al., 1998), this result shows that the production of recombinant protein in plant cell suspensions is lower than in the whole plant.

The plastid genome contains many promoters (Liere and Maliga, 2001), but the strong sigma70-type rRNA operon (Prrn) promoter, which is well-recognized by the plastid-encoded plastid RNA polymerase, is commonly used for plastid transformation (Maliga, 2004). Prrn, a constitutive promoter in chloroplasts and prokaryotes, is reported to cause high levels of transcript accumulation in plastids of plants, but it has been described as having low activity in plant cell suspensions (Michoux et al., 2011). Other factors might also be responsible for lower reteplase production in the obtained cell cultures, such as less differentiated cells and plastids (Michoux et al., 2013), instability of the recombinant protein secreted to the medium due to protease release from disrupted cells (Hong et al., 2002), or aggregation occurring when the daughter cells do not separate in the cell division (Santos et al., 2016). Nevertheless, the rtPA yield was significantly higher than the tPA production (up to 0.017%) in a hairy root system (Kang et al., 2011).

Unlike tPA, reteplase has no carbohydrate side chains and thus can be produced in *E. coli* cells, but most of the protein is present as an inclusion body, making its extraction

and renaturation a time-consuming downstream process, involving solubilization and protein refolding and dialysis (Khodabakhsh et al., 2013). In contrast, as the target protein in our biotechnological system was tagged with 6-his, it was easily purified using a column containing TALON-Superflow® medium precharged with cobalts ions, and the active K2S protein was recovered according to the method previously developed by Abbdoli Nasab et al. (2016).

For the quantitative measurement of reteplase activity, the extracted native protein was obtained from a cell suspension growing in light, since the production was higher than in darkness. The crude extract was purified and concentrated as described in Material and Methods, and the use of the Plasminogen Activator Human Chromogenic Activity Assay Kit allowed activity against plasminogen to be measured throughout 9 h of incubation at 37°C. The behavior of the extracted enzyme (1460 µg) was comparable to the 2.5 UI mL^{-1} tPA standard curve (Fig. 6).

Further approaches to enhance the productivity of the K2S tobacco cell suspensions could be based on modifying the promoter and vector regulatory elements (Hong et al., 2002). Adding signal peptides like 33KDsp may improve secretion efficiency (Haung et al., 2015), minimize processing time and avoid proteolytic and oxidative degradation of recombinant proteins (Fischer, 1999). Fusion tags including fungal hydrophobin (Reuter et al., 2014) or zein-derived peptides could increase the protein accumulation (Joseph et al., 2012). The culture medium could be optimized, e.g. by increasing aeration (Liu and lee, 1999) or adding growth medium supplements such as amino acids (Fischer et al., 1999), gibberellic acid, haemin (Tsoi and Doran, 2002), a carbon source (Santos et al., 2016), protease substrates like gelatin, biopolymers (Lee et al., 2002, Kwon et al., 2002) or bovine serum albumin (James et al., 2000; Baur et al., 2005). The results obtained demonstrate that the extraction of reteplase in native conditions preserves its biological

activity and the biotechnological system could be successfully used for the production of this biopharmaceutical.

4. Conclusions

In this study we developed tobacco cell cultures derived from transgenic K2S plants for the viable production of the biopharmaceutical reteplase. Although the risk of transgene dispersion in transplastomic plants is low due to the maternal mode inheritance, the cell culture system guarantees transgene containment. Also, like other bioreactor systems, the target compounds can be produced under strictly controlled culture conditions, thus avoiding the risk of contamination with human pathogens associated with mammalian cells; it also has the capacity for proper protein folding. We have demonstrated the capacity of the transplastomic K2S cell cultures to produce active reteplase and the enhancing effects of light on its production, achieving a TSP content of 0.277 % after a 2-week growth period. These results open a new avenue for the production of reteplase in transplastomic plant cell cultures as an alternative bio-sustainable system for biopharmaceutical production.

Author contributions

J.P., **P.C.** and **R.C.**, conceived and supervised the experiments. **M.A.**, obtained K2S plants. **R.B.-M.**, performed and developed the method for Mass Spectrometry. D.H., obtained the derived cell lines from K2S plants; developed the in vitro cultures, characterized and quantified the recombinant protein helped by **R.B-M**, and determine the reteplase activity. **J.P.**, **P.C.** and **D.H.**, wrote the manuscript. All authors contributed to results interpretation and reviewed the manuscript.

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Table 1. Sequences of the primers used to amplify the genes by qPCR

Gene	Primer Sequence	Amplicon size bp	Reference
Elongation factor (EF1)	FW: TGGTCAGGAGATTGCGAAAGAGC RV: ACGCAAAACGCTCCAATGGTG	130	Hidalgo et al. (2007)
accD	FW ACAACTGGTGGAGTGACAGC RV ATGCAATGTAGGCGTTGGGT	76	This work
K2S	FW: GCATGACTTTGGTGGGCATC RV: CGGGACATCCTTCTGTCCAC	59	This work

Table 2. Evaluation of housekeeping stability by CV

	Mean Cp		
Housekeeping	EF1	EF1&accD	accD
Light	26.974232	21.374679	15.775127
	28.009782	22.110455	16.211128
	27.866260	21.834132	15.802004
Darkness	27.315741	22.310272	17.304803
	28.948752	23.314494	17.680236
	28.130729	22.747606	17.364483
Mean	27.874249	22.281940	16.689630
SD	0.687129	0.683648	0.856542
CV	0.0247	0.0307	0.0513

Figure legends:

Figure 1. Appearance of the callus cultures (A) and the derived cell suspensions (B) growing under dark and light conditions. (C) Analysis of the relative K2S gene expression normalized to Elongation Factor 1α.

Figure 2. A) Growth capacity of the cell suspension measured as growth index (GI) and biomass productivity (rx) and B) reteplase production of the cell suspensions growing under dark and light conditions. TSP, total soluble protein. Each value is the average of 3 biological replicates ± SE.

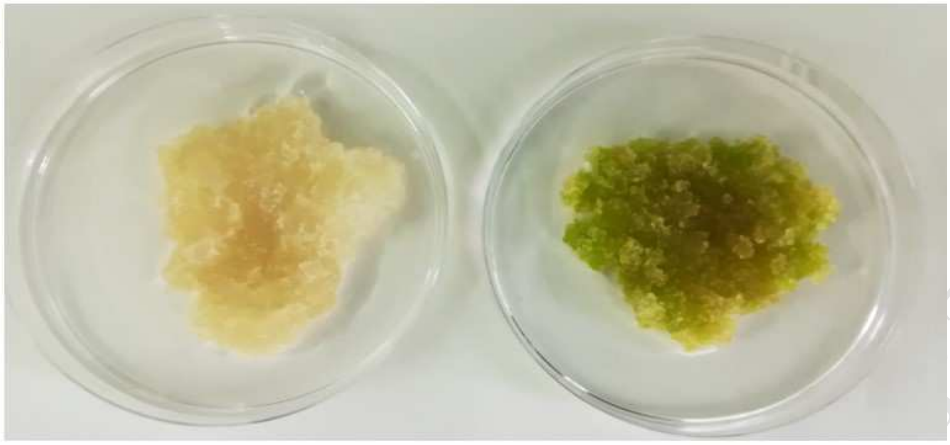
Figure 3. Mass spectrum and fragment ion of the 3 proteotypic peptides for the human tPA protein.

Figure 4. Multiple sequence alignment of tPA in related species. Those that are highlighted represent the seven peptides for method detections and those that are framed correspond to proteotypic peptides for human tPA (protein ID P00750).

Figure 5. Peptide characterization. (A) Identification of the most intensive peptides in cell cultures under dark and light conditions. (B) Chromatogram, indicating specific retention time and intensity of each fragment ion.

Figure 6. Quantitative determination of the native reteplase protein activity, extracted from the K2S cell suspension measured with Tissue type Plasminogen Activator Human Chromogenic Activity Assay Kit.

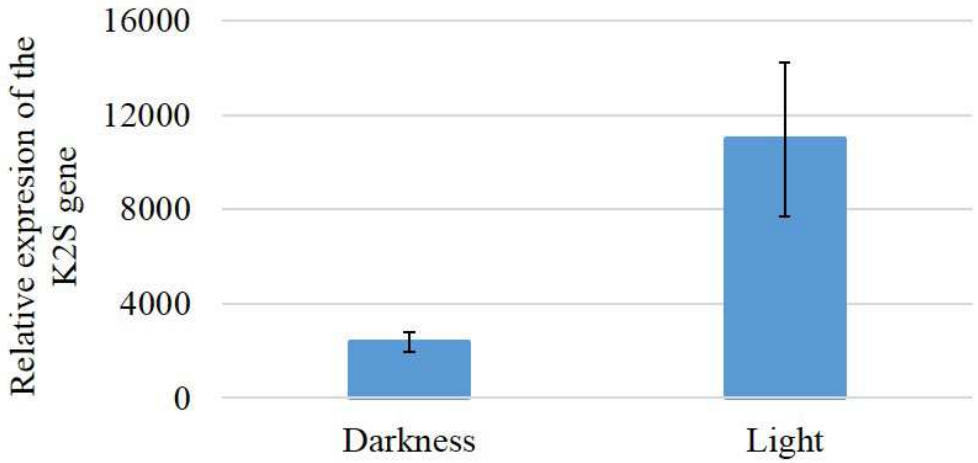
A



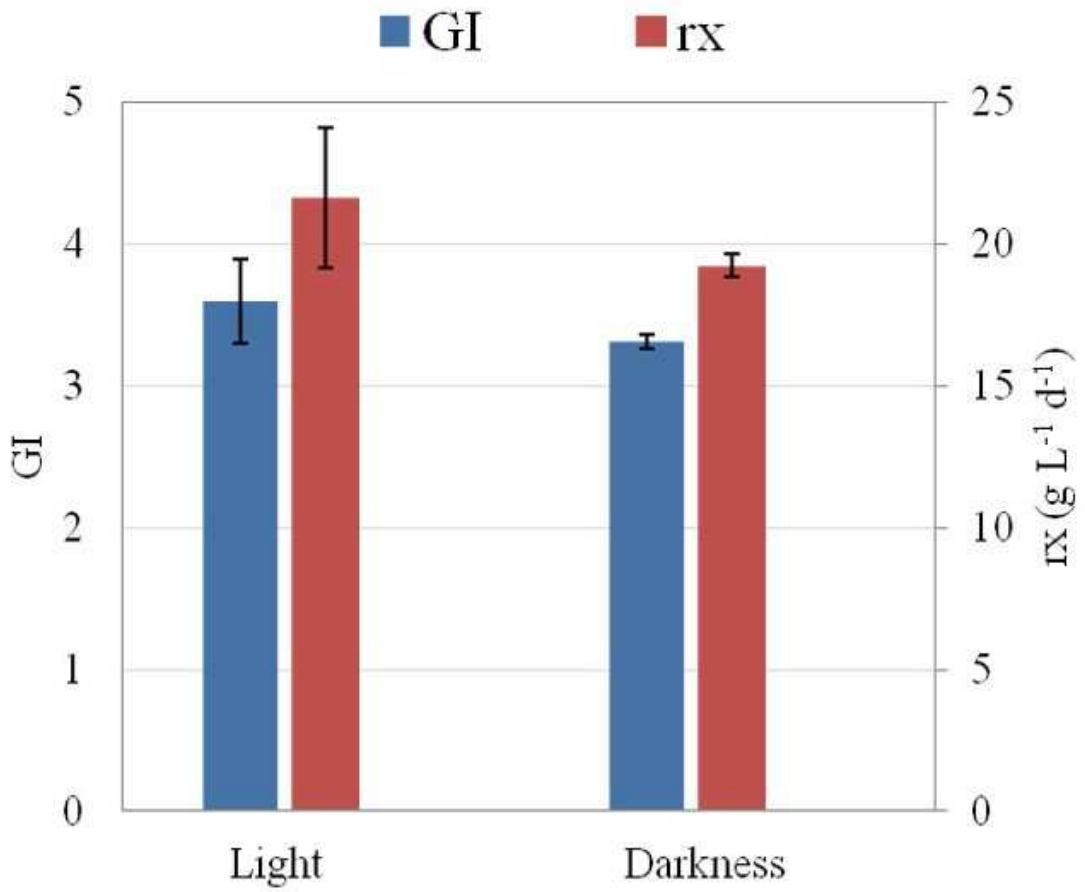
B


500ml

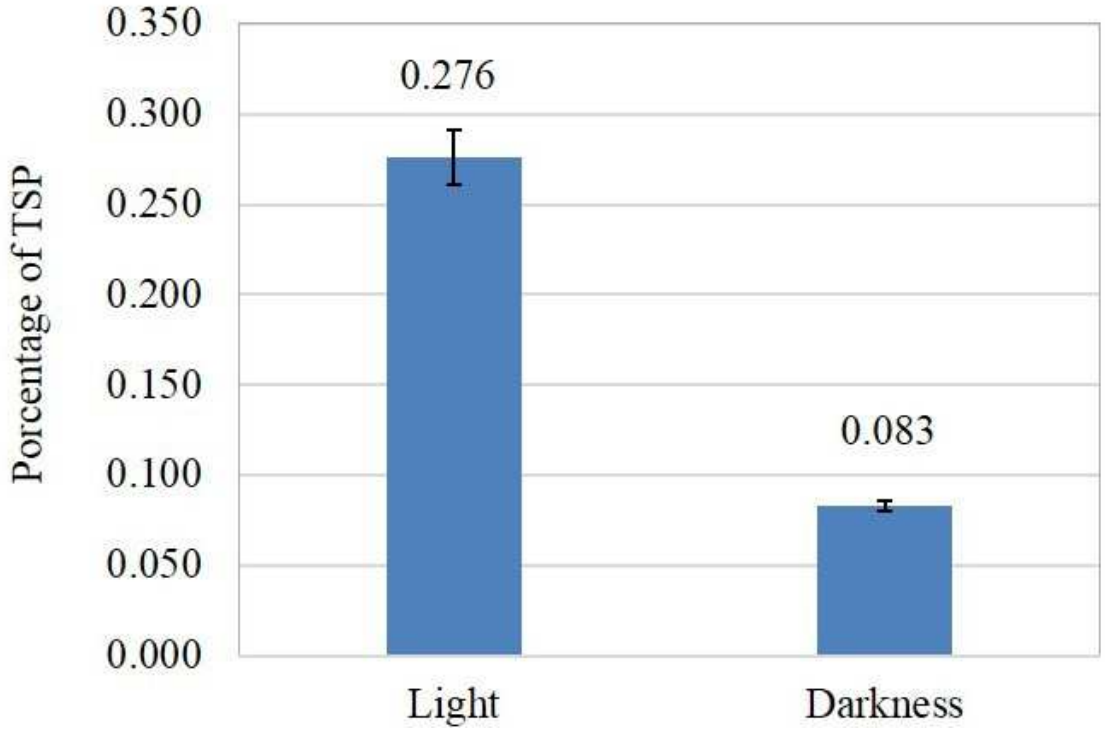
C


Relative expresion of the K2S gene
16000
12000
8000
4000
0
Darkness
Light

A


GI
rx
5
4
3
2
1
0
25
20
15
10
5
0
GI
rx (g L-1 d-1)
Light
Darkness

B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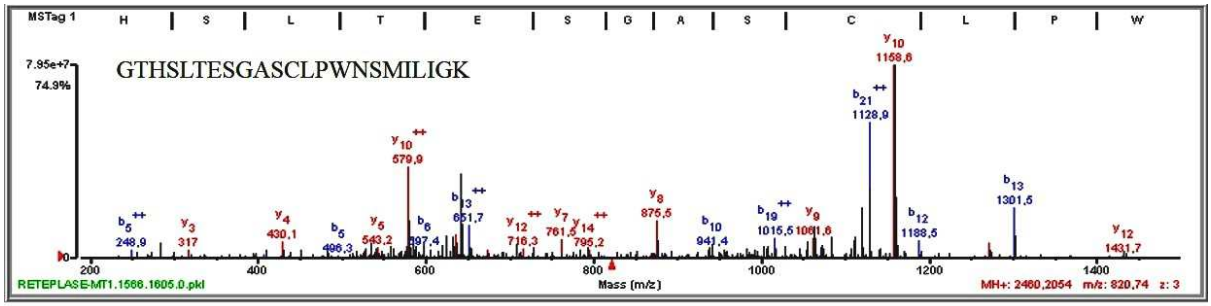
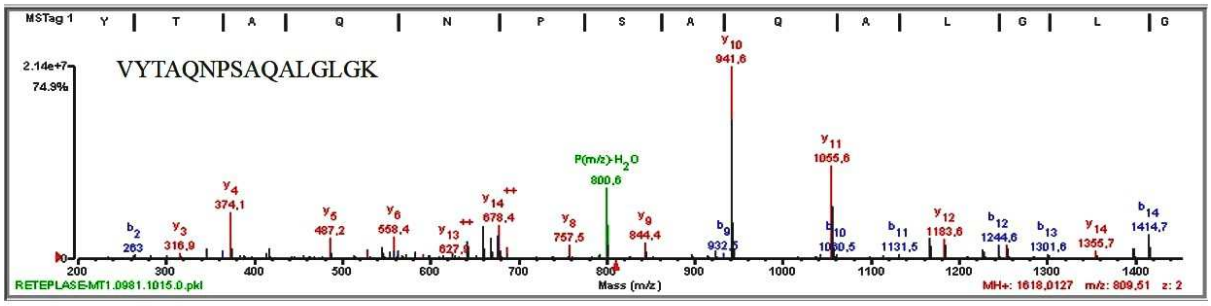
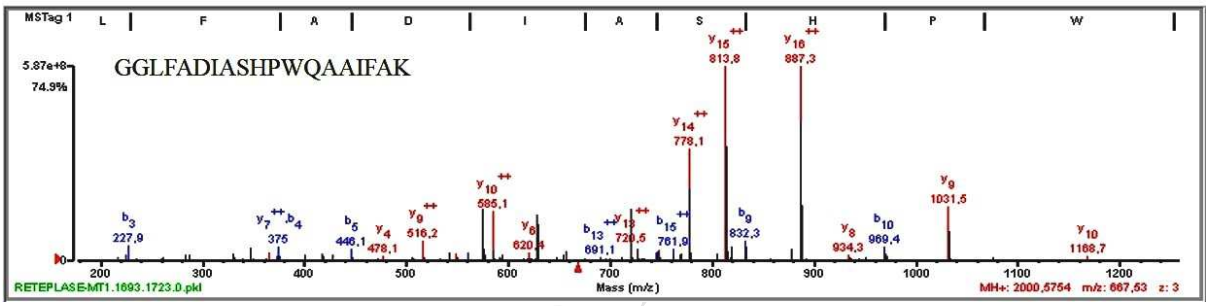
0.083
Light
Darkness







ID	Sub Group #	Length	Identical AA's	%ID	Species	Protein Name
Q28198	1	566	566	100	BOVIN	Tissue-type plasminogen activator
Q8SQ23	1	562	468	83.3	PIG	Tissue-type plasminogen activator
P00750	1	562	460	81.9	HUMAN	Tissue-type plasminogen activator
Q5R8J0	1	562	458	81.5	PONAB	Tissue-type plasminogen activator

```
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P00750 (1) -MDAMKRGLCCVLLLCGAVFVSPSQEIHARFRRGARSYQVICRDEKTQMIYQQHQSWLRPVLRSNRVEYCWCNSGRAQCHSVPVKSCSEPRCFNGGTCQQ
Q5R8J0 (1) -MNAMKRGLCCVLLLCGAVFALPSQEIHARVRRGARSYQVICRDEKTQMIYQQHQSWLRPVLRSNRVEYCWCNSGRAQCHSVPVRSCSEPRCFNGGTCQQ

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Q8SQ23 (1) AIYFSDFVCQCPVGFIGRQCEIDARATCYEDQGITYRGTWSTTESGAECVNWNTSGLASMPYNGRRPDAVKLGLGNHNYCRNPDKDSKPWCYIFKAEKYS
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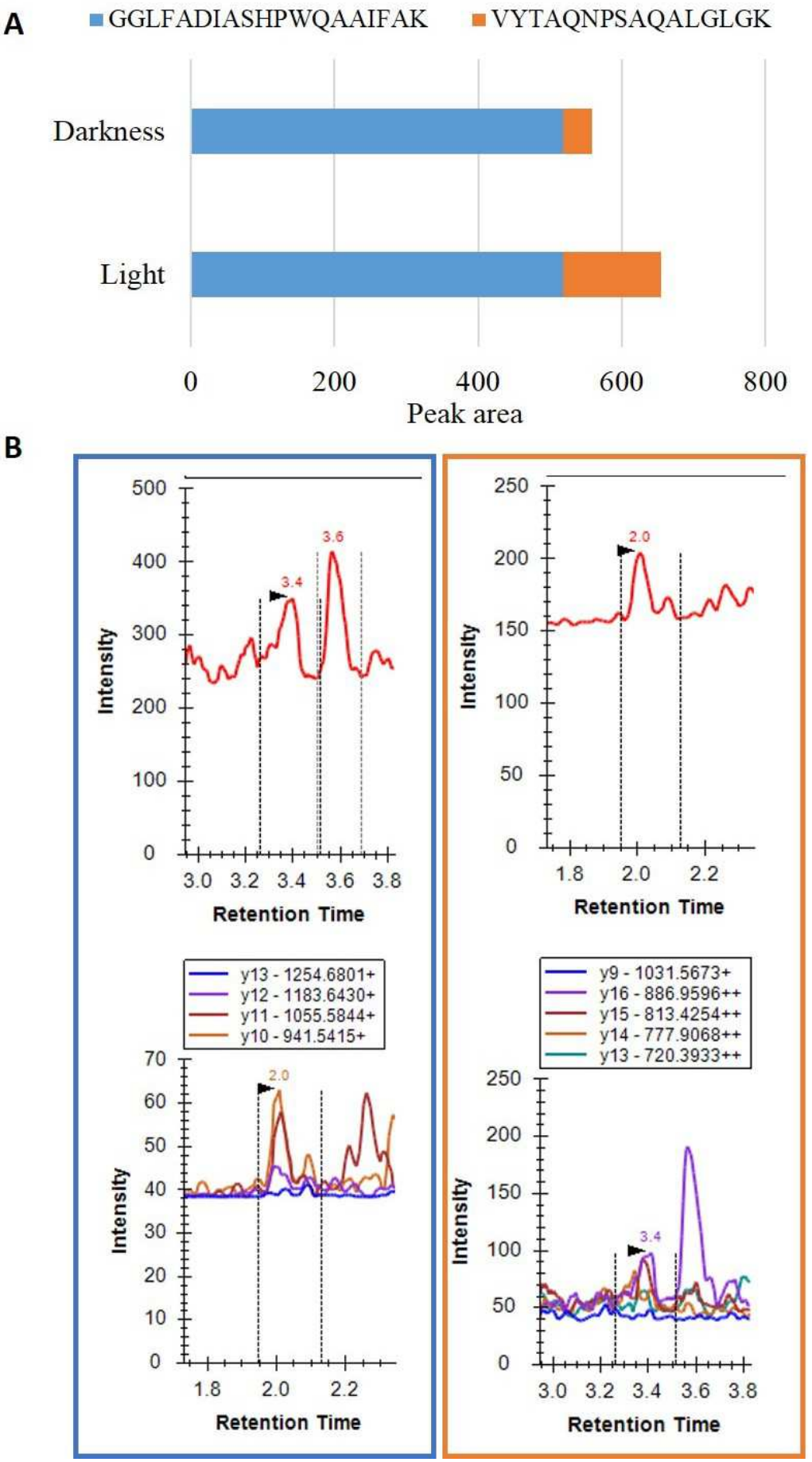
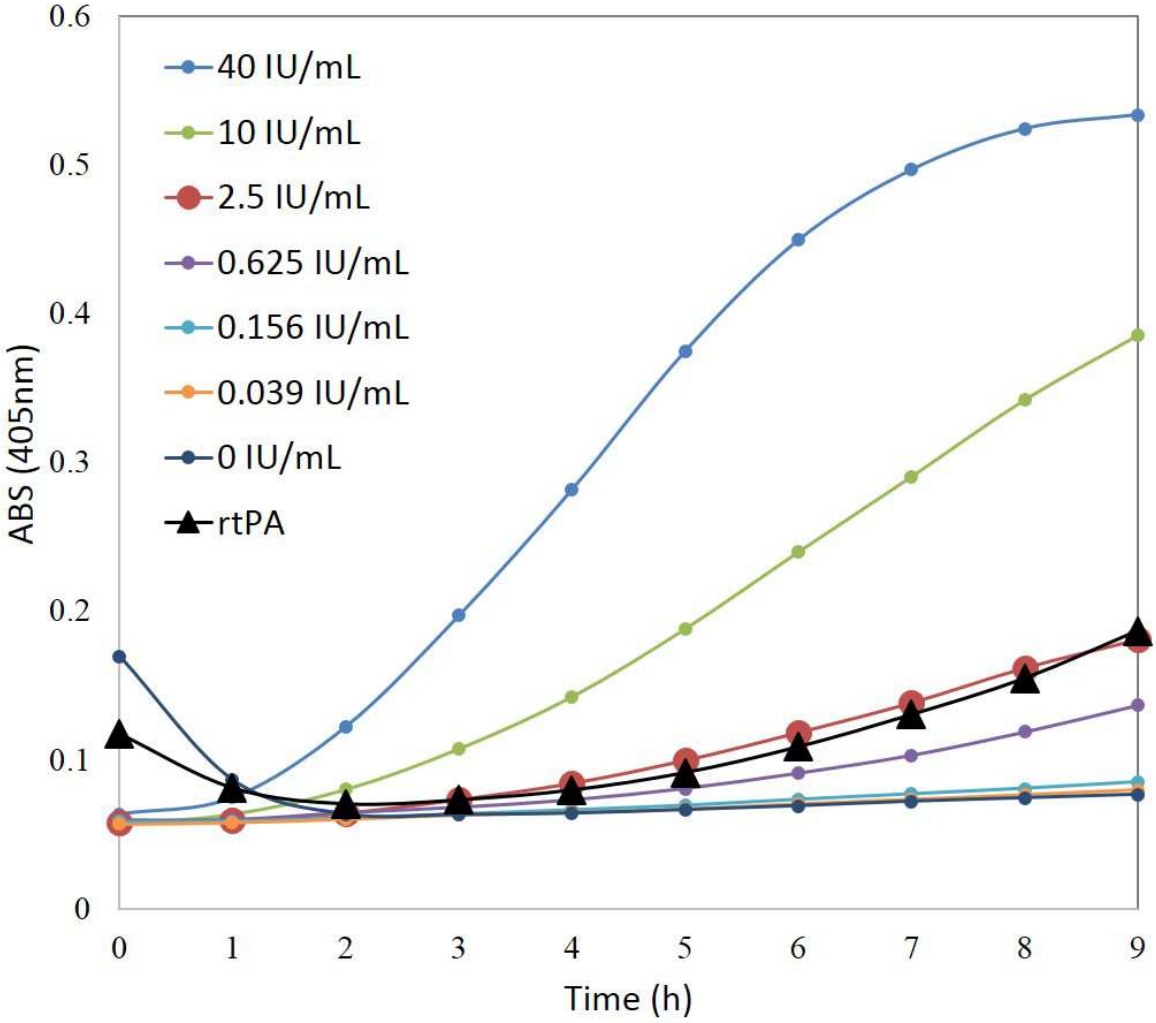

A
GGLFADIASHPWQAAIFAK
VYTAQNPSAQALGLGK
Darkness
Light
0
200
400
600
800
Peak area
B
Intensity
Retention Time
3.4
3.6
2.0
y13 - 1254.6801+
y12 - 1183.6430+
y11 - 1055.5844+
y10 - 941.5415+
y9 - 1031.5673+
y16 - 886.9596++
y15 - 813.4254++
y14 - 777.9068++
y13 - 720.3933++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0.5
0.4
0.3
0.2
0.1
0
ABS (405nm)
40 IU/mL
10 IU/mL
2.5 IU/mL
0.625 IU/mL
0.156 IU/mL
0.039 IU/mL
0 IU/mL
rtPA
0
1
2
3
4
5
6
7
8
9
Time (h)

Highlights:

Reteplase was produced in cell cultures derived from K2S transplastomic tobacco plants.

Heterologous rtPA protein was successfully characterized by mass spectrometry.

Purified native reteplase showed activity against plasminogen.

Reteplase production in the K2S cell cultures was enhanced by light.

Author contributions

J.P., **P.C.** and **R.C.**, conceived and supervised the experiments. **M.A.**, obtained K2S plants. **R.B.-M.**, performed and developed the method for Mass Spectrometry. D.H., obtained the derived cell lines from K2S plants; developed the in vitro cultures, characterized and quantified the recombinant protein helped by **R.B-M**, and determine the reteplase activity. **J.P.**, **P.C.** and **D.H.**, wrote the manuscript. All authors contributed to results interpretation and reviewed the manuscript.